Monteiro, F., Bernal, V., Chaillet, M., Berger, I., & Alves, P. M. (2016). Targeted supplementation design for improved production and quality of enveloped viral particles in insect cell-baculovirus expression system. *Journal of Biotechnology*, *233*, 34-41. https://doi.org/10.1016/j.jbiotec.2016.06.029

# TARGETED SUPPLEMENTATION DESIGN FOR IMPROVED PRODUCTION AND QUALITY OF ENVELOPED VIRAL PARTICLES IN INSECT CELL-BACULOVIRUS EXPRESSION SYSTEM

Francisca Monteiro[1,2], Vicente Bernal[3+], Maxime Chaillet[4,5], Imre Berger[4,5,6*] and Paula M. Alves[1,2*]

[1] iBET, Instituto de Biologia Experimental e Tecnológica, Oeiras, Portugal;
[2] Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa , Oeiras, Portugal;
[3] Departamento de Bioquímica y Biología Molecular B y Imunología. Facultad de Química. Campus Internacional de Excelencia "Mare Nostrum". Universidad de Murcia, Murcia, Spain;
[4] The *European Molecular Biology Laboratory*, Grenoble, France;
[5] Unit of Virus Host Cell Interaction UVHCI, Université Grenoble Alpes – EMBL – CNRS Unité Mixte de Recherche Grenoble, France ;
[6] The School of Biochemistry, University of Bristol, Bristol BS8 1TD, UK;
[+]Current address: Área de Biología. Dirección de Nuevas Energías. Centro de Tecnología de REPSOL, Autovía A-5, Km 18, 28935 Móstoles-Madrid, Spain.

**Correspondence:**

- [*]Prof. Paula M. Alves (marques@itqb.unl.pt);

**Phone:** +351-21-4469362
Animal Cell Technology Unit, IBET/ITQB, Apartado 12, 27801-901 Oeiras, Portugal

- [*]Prof. Imre Berger (iberger@embl.fr);

**Phone:** +33-4-7620 7061
EMBL, 6 Rue Jules Horowitz, 38042 Grenoble CEDEX 9, France

**Abstract**

The recent approval of vaccines and gene therapy products for human use produced in the Insect Cell-Baculovirus Expression Vector System (IC-BEVS) underlines the high potential and versatility of this platform. The interest in developing robust production processes emerges to cope with manufacturing pressure, as well as stringent product quality guidelines. Previously, we addressed the impact of the baculovirus infection on the physiology of insect host cell lines, identifying key cellular pathways enrolled in heterologous gene/protein expression. In the present work, this knowledge was applied to design tailored media supplementation schemes to boost IC-BEVS production yields and quality of enveloped viral particles: influenza VLPs (Inf-VLP) and baculovirus vectors (BV).

The addition of reduced glutathione, antioxidants and polyamines increased the cell specific yields of baculovirus particles up to 3 fold. Cholesterol was identified as the most critical system booster, capable of improving 2.5 and 6-fold cell specific yields of BV and Inf-VLPs, respectively. Surprisingly, the combination of polyamines and cholesterol supplementation improved baculovirus stock quality, by preventing the accumulation of non-infectious particles during viral replication while selectively increasing infectious particles production. In addition, the specific yields of both enveloped viral particles, BVs and Inf-VLPs, were also increased.

The correlation between supplement addition and systems productivity was extensively analyzed, providing a critical assessment on final product quantity and quality as drivers of bioprocess optimization efforts.

**Key-words:** Baculovirus-insect cell system; metabolism; productivity; product quality; bioprocess optimization; VLPs

## 1. Introduction

The insect cell-baculovirus expression system (IC-BEVS) is now on the frontline of both pharmaceutical and biotechnological fields after the recent approval of several human therapeutics produced in this system, namely Cervarix®, Flublok® and Glybera® (Cox and Hollister, 2009; Lowy and Schiller, 2006; Moran, 2012). As the market of IC-BEVS based biopharmaceuticals progresses (Lu et al., 2012; Ylä-Herttuala, 2012), the interest in developing high-titer and robust production processes is expected to rise.

Molecular biology studies have provided exciting discoveries on baculovirus–host interactions (reviewed in Monteiro et al., 2012). However, the biological constraints that govern baculovirus infection in the biotechnological context are poorly understood. The analysis of cell culture parameters and media components influencing productivity has turned possible the implementation of high cell density bioprocesses with increased and sustained production of

recombinant proteins *via* BEVS (Bédard et al., 1997; Chan et al., 1998; Chiou et al., 2000). The combination of metabolic flux analysis with the rational design of a feeding strategy improved baculovirus yields (6- to 7-fold) in high cell density cultures (Carinhas et al., 2010). Also, the on-line monitoring of the oxygen uptake rate (OUR) in baculovirus infected insect cells supported the design of feeding strategies able to boost up to 13 times the recombinant protein yields (Palomares et al., 2004). Overall, the successful application of metabolic and bioprocess engineering strategies to the IC-BEVS shows that there is room for improvement. Also, it seems clear that knowledge on key parameters of cellular physiology can help in devising such efforts towards increased productivity.

Although progress has been made in tuning the baculovirus to accommodate the expression of challenging targets (Bieniossek et al., 2012; Palmberger et al., 2013), the accumulation of defective interfering particles (DIF) with increasing viral passages constraints baculovirus-based bioprocesses (Pijlman et al., 2001). The loss of expression of heterologous gene(s) and low infectivity of the baculovirus expression vector occurs as a consequence of recombination events during viral replication (Pijlman et al., 2003), and the impact of the heterologous gene(s) on this event is not fully understood.

In previous works, metabolic pathways correlated with baculovirus infection and IC-BEVS productivity were identified based on fluxome analysis (Bernal et al. 2009, Monteiro et al. *in preparation*) and metabolomic fingerprinting (Monteiro et al. 2014). Although several metabolic differences were assessed between the two insect cell lines more used for protein and virus production (*Trichoplusia ni* High Five and *Spodoptera frugiperda* Sf9 cells, respectively) responses to infection were very similar (Monteiro et al. 2014).

In the present work, we designed culture supplementation schemes aiming to boost IC-BEVS productivity and quality of complex enveloped viral particles: influenza VLPs (Inf-VLPs) and baculovirus (BV). Supplements selection was based on our previous identification of the metabolic pathways correlated with IC-BEVS productivity (Monteiro et al., 2014), with the goal of enhancing pathway activity and systems performance. An orthogonal screening of culture conditions was performed to pin-point the culture setting leading to maximal productivity in Sf9 cells, addressing target specific key requirements. Finally, an optimized bioprocess for the production of high quality enveloped viral particles *via* BEVS was implemented.

## 2. Materials and methods

### 2.1. Cell lines and culture maintenance

*Spodoptera frugiperda* derived Sf9 cell line was obtained from the European Collection of Cell Cultures (No. 89070101, ECCAC). Cells were maintained in serum- and protein-free Sf900II insect cell culture medium (Gibco, Glasgow, UK) in 500 mL Erlenmeyer flasks

(Corning, USA) with 50 mL working volume. Cultures were kept in a humidified incubator operated at 90 rpm and 27ºC. Routinely, Sf9 cells were re-inoculated every 3-4 days at $0.45x10^6$ cells.$mL^{-1}$. Cell concentration was determined by hemocytometer cell counts and viability evaluated by the trypan blue exclusion method.

### 2.2. Baculoviruses and viral stock preparation

The recombinant *Autographa californica* nucleopolyhedrovirus BvHA5M1 was used throughout the work as the expression vector of the enveloped influenza VLPs (Inf-VLPs). This vector is a dual baculovirus, encoding two influenza genes: Hemagglutinin type 5 (H5), under the control of the polyhedrin promoter, and matrix protein 1 (M1), under the control of the p10 promoter.

BvHA5M1 was amplified by infecting Sf9 cells at $1x10^6$cells.$mL^{-1}$ with a multiplicity of infection (MOI) of 0.01 IP.$cell^{-1}$ in a 10 L bioreactor (ED10, Sartorius AG, Goettingen, Germany). To generate a highly concentrated viral stock, a polyethylene glycol (PEG)-based concentration process was applied. Briefly, virus-containing culture supernatant was mixed with 8.5% (v/v) of a sterile PEG solution prepared in phosphate buffer saline (PBS), and incubated overnight at 4ºC. The mixture was centrifuged at 3200xg for 30 min at 4ºC, and the collected pellet containing the baculovirus was suspended in 0.5 M sucrose. The concentrated viral stocks were titrated, aliquoted and kept at -80ºC until further use.

### 2.3. Baculovirus titration and total particles quantification

Baculovirus infectious particles quantification was performed following the MTT assay, as previously described in Roldão et al. (2009). Baculovirus total particles concentration was assessed by counting the baculovirus particles in a Nanosight NS500 (Nanosight Ltd., Salisbury, UK), using the Nanoparticle Tracking Analysis (NTA) software. The average size of purified Inf-VLPs samples and baculovirus was identified *a priori* for the determination of the gates for both particles quantification (data not shown). To exclude minor errors due to counting of cellular debris, exosomes, and other particles that can interfere with the accuracy of the measurements, appropriate controls were performed (supernatant of non-infected cultures at the correspondent cell densities). The measurements were performed at least in triplicates with a typical standard deviation (SD) below 20%.

### 2.4. Supplements preparation

The culture supplements tested, listed in Table 1, were selected as boosters of the metabolic pathways correlated with IC-BEVS productivity. Supplements concentration was set taking into account the manufacturer instructions and preliminary assays of their impact in the specific productivity and viability of Sf9 cells (data not shown). Supplements were prepared in Sf900II insect culture media (Gibco), and stored according to the manufacturer instructions. Cholesterol supplementation was performed in combination with 0.4 mg.$L^{-1}$ albumin (Merck Millipore, Billerica, MA, USA) as a carrier.

### 2.5. Cell growth, infection and production studies

The experimental set-up is depicted in Figure 1. To identify the impact of the supplementation strategy on enveloped viral particles yields, an exploratory screening was designed using the Advanced Microscale Bioreactor (Ambr, model AMBR24c) culture system (TAP Biosystems, Cambridge, UK) (Fig. 1A) previously customized to be suitable for IC-BEVS expression experiments (Berger et al., 2013a, 2013b). Importantly, a cooling element was installed to operate at the temperatures relevant for insect cell production (26-27ºC). The customized Ambr robot was placed in a tailor-made plastic containment for sterile operations. The oxygen was provided by compressed and filtered air, supplied by a tubing connected to the robot, avoiding costly pure air installation requirement. From the experimental design, 12 single use bioreactors without sparger tubings were inoculated manually with 13 mL of Sf9 cultures and the corresponding supplements (Fig 1). Sf9 cells were inoculated at $0.5x10^6$ cells.mL$^{-1}$, and infected at approximately $1x10^6$ cells.mL$^{-1}$ with MOIs of 0.2, 1 and 5 IP.cell$^{-1}$. Each bioreactor was equipped with dissolved oxygen (DO) and pH sensors, automatically recording and monitoring each individual bioprocess. Culture agitation was performed by an 11.2 mm impeller, set at 1400 rpm. In order to provide sufficient oxygen to the cultures, an additional air input with a flowrate up to 0.9 mL/min was automatically provided to avoid the DO percentage to drop below 40%. In practice, the latter never dropped below 70%.

To disclose specific requirements of each target in Sf9 host cell line, an extensive screening in 100 mL Erlenmeyers (Corning, USA), with 10 mL working volume, was performed (Fig. 1B). Sf9 cells were inoculated at $0.5x10^6$ cells.mL$^{-1}$, kept at 27ºC and infected at $1x10^6$ cells.mL$^{-1}$ with MOIs of 0.2, 1 and 5 IP.cell$^{-1}$. Additionally, to evaluate if synergies between supplements would occur, the combination of culture supplements was performed at this same scale, using a MOI of 0.2 IP.cell$^{-1}$(Fig. 1C).

The best culture condition leading to higher cell specific productivities of enveloped viral particles was validated in a 0.5 L stirred tank bioreactor (BIOSTAT® QPlus, Sartorius AG, Goettingen, Germany) (Fig. 1D). DO was set to 30% of air saturation and controlled by sequential $N_2$-stirring-$O_2$ cascade mode with 0.01 vvm gas flow rate. Temperature was kept at 27ºC and the operation was performed within a stirring range of 90-180 rpm. Sf9 cells were inoculated at $0.5x10^6$ cells.mL$^{-1}$ and infected with BvHA5M1 at an MOI of 0.2 IP.cell$^{-1}$, 24 hours after inoculation.

For all culture settings (Fig. 1A-D), supplements were added in a two-times addition mode, at inoculation, in order to precondition cells enabling an adaptation phase to their addition, and at infection, to promote a productive cellular state, medium concentrations are detailed in Table 1. Non-supplemented cultures infected with the same set of MOIs were performed as controls. Samples were collected every 24 hours and cell concentration and viability assessed. To evaluate productivity, samples were collected at 48 hours post-infection

(hpi), the time after which the expression from polyhedrin and p10 promoters is maximal. In the bioreactor cultures, enveloped viral particles production was followed throughout the entire process (up to 96 hpi).

### 2.6. Influenza VLPs quantification

The quantification of the influenza VLPs (Inf-VLPs) was performed by solid phase sandwich ELISA (SEK002, Sino Biological Inc., Beijing, China) specific for the hemagglutinin (HA) displayed on the surface of the particles. The assay was done according to manufacturer instructions, and HA-containing particles quantified in culture supernatant samples.

### 2.7. Product stability assays in supplemented media

Samples of BVs in culture media were incubated with the supplements at the same concentration added to the cultures (Table 1). Stability assays were performed at 27ºC during 48 hours, and supplement effect on baculovirus stability was evaluated by comparing virus infectivity before and after incubation. Appropriate controls were performed, subjected to the same incubation time in non-supplemented media.

### 2.8. Statistical analysis

Hypothesis testing was performed using Student's t-test. A 95% confidence interval was considered to be statistically significant.

## 3. Results

### 3.1. Media manipulation strategies affect cell growth without compromising cell viability and product stability

Ideally, medium supplements would improve cell productivity without compromising cell growth and product stability. The effect of culture supplements on the growth rate of Sf9 cells is presented in Table 2. GSH, antioxidants, polyamines and biotin significantly decreased the specific growth rate up to 2 fold, whereas cholesterol impaired cellular growth. Cell viability was not compromised by the supplement addition (data not shown) and baculovirus stability was not significantly affected (Supplementary fig. 1).

### 3.2. Media manipulation strategies boost productivity of enveloped viral particles in Sf9 cells

The exploratory screening using the Ambr culture system showed that baculovirus and Inf-VLPs yields increased (Supplementary Figure 2) in Sf9 cells following supplementation. These results encouraged an extensive screening of the listed culture supplements (Table 1) and also the evaluation of the supplementation impact on product quality.

The production of enveloped viral particles increased up to 3-fold by supplements with redox balancing properties, mainly GSH, antioxidants and polyamines (Fig. 2). Although this set of supplements decreased cell growth rate, their boost on cell specific productivity of infectious BVs was high enough to achieve higher volumetric productivities and cell specific yields when

compared to control cultures (Supplementary Table 1). Cholesterol supplementation boosted both BVs and Inf-VLPs cell specific yields by 2.5 and 6-fold, respectively (Fig. 2A and B), which is a noteworthy result given that cholesterol halted culture growth (Table 2). In general, higher improvements on BVs production were achieved in infections performed with medium-high MOIs, yet for Inf-VLPs we could not trace a clear correlation between fold-change in the production and MOI used (Fig. 2).

### 3.3. Media manipulation strategies improve the quality of baculovirus particles produced in Sf9 cells

The baculovirus used throughout this work is a difficult to produce virus, since relatively low titers were reached upon virus stock preparation. Thus, this baculovirus is a good candidate to test our proof-of-principle and test whether these supplements could also affect product quality. To evaluate the effect of tested supplements on product quality we assessed the ratio of infectious (IPs) to total particles (TPs). Viral quality was defined as a direct measure of infectivity, meaning IPs generated. This analysis was performed in low MOIs infected cultures, since those conditions are recommended for virus amplification towards higher viral stock quality.

Polyamines and cholesterol yielded proportional improvements of both TPs and IPs, resulting in similar TPs/IPs ratios (Fig. 3, upper panel). Importantly, GSH and antioxidants combined with polyamines supplementation increased the IPs levels without altering TPs formation (Fig. 3, bottom panel). Therefore, these supplements improved final stock quality, as demonstrated by the decrease in the TPs/IPs ratio (Fig. 3, upper panel). These results show that, in addition to productivity, final product quality is also enhanced with the tested supplements.

### 3.4. Synergistic combination of supplements improve the production of enveloped viral particles in Sf9 cells

The best supplements were combined in groups of metabolic related pathways, *i.e.* oxidative stress and lipid metabolism and their impact on the specific productivity of Sf9 cells was evaluated. The correlation between MOI and supplements impact on productivity was not straightforward and depended on the target produced (Fig. 2). Since high MOI infections at larger scales can be limiting and we are working with a challenging virus regarding infectious particles titers, we decided to proceed with low MOI infections to analyze the synergies between the supplements. Infections were performed at low MOI and the production of BVs and Inf-VLPs assessed (Fig. 4). The combination of cholesterol with polyamines boosted the specific yields of BVs by 7-fold, twice the value obtained by the addition of cholesterol alone, while no beneficial effect was observed in the Inf-VLPs specific yields by adding up polyamines.

### 3.5. Implementation of an optimal bioprocess for the production of enveloped viral particles in Sf9 cells *via* BEVS

The results obtained with cholesterol and polyamines supplementation were validated in 0.5 L stirred-tank bioreactors (Fig. 5). The addition of both supplements increased the production of infectious BVs (Fig. 5A) and Inf-VLPs (Fig. 5B) compared to non-supplemented cultures. Additionally, virus quality was also improved since the ratio between TPs and IPs was lower in the supplemented cultures, and the virus amplification factor was enhanced by 6 fold (Table 3).

## 4. Discussion

Several reports on IC-BEVS bioprocess optimization describe fed-batch strategies to improve the production of recombinant proteins (Nguyen et al., 1993; Yang et al., 1996; Taticek and Shuler, 1997; Chan et al., 1998; Palomares et al., 2004) or non-enveloped viral vectors ( Liu et al., 2010; Mena et al., 2010). Even though the IC-BEVS is massively used for the production of VLPs (Liu et al., 2013), and efforts have been made in understanding which are the process parameters that contribute for better VLP yields (Cruz et al., 1998; Maranga et al., 2002; Palomares et al., 2012; Pillay et al., 2009; Vieira et al., 2005), reports on the identification of the metabolic constraints in such targets production are still scarce. This is especially critical in the case of enveloped viral particles produced *via* BEVS, challenging complex products with stringent quality requirements not yet fully understood. In the present work, we analyzed the impact of cell culture supplements in IC-BEVS productivity, focusing on enhancing quality and titers of enveloped viral particles. The rationale behind this approach was provided by our previous metabolomic characterization of the system, where metabolic pathways correlated with baculovirus replication and productivity were identified (Monteiro et al., 2014). The supplements tested were selected as boosters of these metabolic pathways, with the final goal of driving cellular performance towards a higher productivity phenotype.

We observed that the addition of GSH, antioxidants and polyamines, increased cell specific yields of infectious baculovirus particles. Baculovirus stock quality, *i.e.* infectivity, is influenced by the metabolic state of the producer cell, as productivity and cellular metabolism are correlated (reviewed in Aucoin et al., 2010). Among several roles in a cell, GSH is involved in reactive oxygen species (ROS) detoxification and protein folding (Chakravarthi et al., 2006). Oxidative stress occurs as a consequence of baculovirus infection (Wang et al., 2001), which can be overcome by over-expressing the antioxidant enzyme manganese superoxide dismutase thus precluding lipid and protein oxidation in baculovirus infected cells (Wang et al., 2004). Besides aiding in nucleic acids stabilization and transcription modulation, polyamines are able to improve membrane rigidity, as well as preventing lipid peroxidation given their antioxidant properties (Wallace et al., 2003). The positive effect of polyamines on the production of enveloped virus has been described (Raina et al., 1981; Rodrigues et al., 2013). Replication of baculovirus vectors is susceptible to the cellular metabolic state (Carinhas et al., 2010, 2009), and as seen here can beneficiate from a less-oxidative cellular microenvironment. Thus, we

hypothesize that the joint action of polyamines and antioxidants, by empowering redox homeostasis, can contribute to the observed improvements on baculovirus yields with increased quality (*i.e.* infectivity).

Although the recommended practices of baculovirus stock management were followed, such as viral amplification at low MOIs and maintenance of the virus working stock at low passage number (Lesch et al., 2011), only low-titer BvHA5M1 virus stocks were achieved. Several factors may explain this effect, like the recombinant construct expressed, which can influence the baculovirus stock titer either by promoting instability of the viral DNA or by being cytotoxic, for instance. We have seen that by simply expressing constructs of different variants of influenza hemagglutinin, the generated viral stocks have significantly different titers, with 10 to 100 fold changes in infectious particles production (data not shown). A correlation between the expressed construct and the baculovirus titer appears to occur, and further work should be performed to disclose this effect.

Cholesterol was the main system booster, capable of improving cell specific yields of both baculovirus and enveloped VLPs. The manipulation of lipid and cholesterol metabolism has culminated in improved production of enveloped viral particles in several producer systems (Cervera et al., 2013; Chen et al., 2010; Mitta et al., 2005; Rodrigues et al., 2009). Cholesterol has an important role in membrane fluidity and rigidity, being invaluable for biogenesis and functionality (Bloch, 1983), as well as aiding in the stabilization of viral particles envelope, maturation and budding (Chan et al., 2010), thus being a major contributor to viral infectivity. Although few works detail the role of lipids in the IC-BEVS, it is recognized that when insect cells are subjected to lipid deprivation cell degeneration occurs, and the production of baculovirus is impaired (Goodwin, 1991). Similarly to wild-type virus, the budding of influenza-derived VLPs from the host cell occurs preferentially at lipid rafts (Chen et al., 2007), *i.e.*, bioactive domains in the plasma membrane enriched in cholesterol and sphingolipids (Simons and Ikonen, 1997). Evidences suggest that baculovirus budding is not restricted to such domains (Zhang et al., 2003), however, the importance of lipidic cytosolic vesicles trafficking during the baculovirus infection cycle was demonstrated (Long et al., 2006; Yuan et al., 2011). Supplementation with lipid components can not only influence host cell metabolism, by assisting lipid overproduction imposed during infection and production of enveloped viral particles, but also influence membrane biogenesis and homeostasis. Taken together, our observations highlight the importance of proper membrane fitness to enable correct folding of proteins that intimately interact with membrane lipids, and the budding of the viral particles thus produced in insect cells.

Given the increasing popularity of the IC-BEVS as a vaccine production platform, the identification of key players in final product quantity and quality is pivotal. Identification of the traits that influence systems' performance can empower us to develop cells with superior

phenotypes, improve target quality and implement more robust bioprocesses. For the IC-BEVS, the maintenance of redox homeostasis and an enhanced cholesterol metabolism are key parameters that should be considered when developing and implementing highly productive bioprocesses. The work herein presented merges fundamentals with applied research, which culminated in the implementation of an IC-BEVS bioprocess that delivers higher quality and quantity of enveloped viral particles.

## 5. Acknowledgments

This work was supported by ComplexINC *FP7/HEALTH.2011.1.1-1/279039* project. Francisca Monteiro acknowledges FCT for her PhD fellowship grant (SFRH/BD/7013/2010).

## 6. References

Aucoin, M.G., Mena, J.A., Kamen, 2010. Bioprocessing of baculovirus vectors: a review. Curr. Gene Ther. 10, 174–186.

Bédard, C., Perret, S., Kamen, A.A., 1997. Fed-batch culture of Sf-9 cells supports 3x10 7 cells per ml and improves baculovirus-expressed recombinant protein yields. Biotechnol. Lett. 19, 629–632.

Berger, I., Chaillet, M., Garzoni, F., Yau-Rose, S., Barney, Z., 2013a. High-Throughput Screening of Multiple Protein Complexes. Am. Lab. 45(8): 32–35.

Berger, I., Garzoni, F., Chaillet, M., Haffke, M., Gupta, K., Aubert, A., 2013b. The MultiBac Protein Complex Production Platform at the EMBL. J. Vis. Exp. 1–8. doi:10.3791/50159

Bieniossek, C., Imasaki, T., Takagi, Y., Berger, I., 2012. MultiBac: expanding the research toolbox for multiprotein complexes. Trends Biochem. Sci. 37, 49–57. doi:10.1016/j.tibs.2011.10.005

Bloch, K.E., 1983. Sterol, Structure and Membrane Function. Crit. Rev. Biochem. Mol. Biol. 14, 47–92. doi:10.3109/10409238309102790

Carinhas, N., Bernal, V., Monteiro, F., Carrondo, M.J.T., Oliveira, R., Alves, P.M., 2010. Improving baculovirus production at high cell density through manipulation of energy metabolism. Metab. Eng. 12, 39–52.

Carinhas, N., Bernal, V., Yokomizo, A.Y., Carrondo, M.J.T., Oliveira, R., Alves, P.M., 2009. Baculovirus production for gene therapy: the role of cell density, multiplicity of infection and medium exchange. Appl. Microbiol. Biotechnol. 81, 1041–9.

Cervera, L., Gutiérrez-Granados, S., Martínez, M., Blanco, J., Gòdia, F., Segura, M.M., 2013. Generation of HIV-1 Gag VLPs by transient transfection of HEK 293 suspension cell cultures using an optimized animal-derived component free medium. J. Biotechnol. 166, 152–65.

Chakravarthi, S., Jessop, C.E., Bulleid, N.J., 2006. The role of glutathione in disulphide bond formation and endoplasmic-reticulum-generated oxidative stress. EMBO Rep. 7, 271–5.

Chan, L.C., Greenfield, P.F., Reid, S., 1998. Optimising fed-batch production of recombinant proteins using the baculovirus expression vector system. Biotechnol. Bioeng. 59, 178–188.

Chan, R.B., Tanner, L., Wenk, M.R., 2010. Implications for lipids during replication of enveloped viruses. Chem. Phys. Lipids 163, 449–459. doi:10.1016/j.chemphyslip.2010.03.002

Chen, B.J., Leser, G.P., Morita, E., Lamb, R.A., 2007. Influenza virus hemagglutinin and neuraminidase, but not the matrix protein, are required for assembly and budding of plasmid-derived virus-like particles. J. Virol. 81, 7111–23.

Chen, Y., Ott, C.J., Townsend, K., Subbaiah, P., Aiyar, A., Miller, W.M., 2010. Cholesterol Supplementation During Production Increases the Infectivity of Retroviral and Lentiviral Vectors Pseudotyped with the Vesicular Stomatitis Virus Glycoprotein (VSV-G). Biochem. Eng. J. 44, 199–207.

Chiou, T.-W., Hsieh, Y.-C., Ho, C.S., 2000. High density culture of insect cells using rational medium design and feeding strategy. Bioprocess Eng. 22, 483–491. doi:10.1007/s004499900091

Cox, M.M.J., Hollister, J.R., 2009. FluBlok, a next generation influenza vaccine manufactured in insect cells. Biologicals 37, 182–9.

Cruz, P.E., Cunha, A., Peixoto, C.C., Clemente, J., Moreira, J.L., Carrondo, M.J., 1998. Optimization of the production of virus-like particles in insect cells. Biotechnol. Bioeng. 60, 408–418. doi:10.1002/(SICI)1097-0290(19981120)60:4<408::AID-BIT2>3.0.CO;2-Q

Goodwin, R.H., 1991. Replacement of vertebrate serum with lipids and other factors in the culture of invertebrate cells, tissues, parasites, and pathogens. In Vitro Cell. Dev. Biol. 27A, 470–478. doi:10.1007/BF02631147

Lesch, H.P., Makkonen, K.E., Laitinen, A., Määttä, A.M., Närvänen, O., Airenne, K.J., Ylä-Herttuala, S., 2011. Requirements for baculoviruses for clinical gene therapy applications. J. Invertebr. Pathol. 107. doi:10.1016/j.jip.2011.05.010

Liu, F., Wu, X., Li, L., Liu, Z., Wang, Z., 2013. Use of baculovirus expression system for generation of virus-like particles: successes and challenges. Protein Expr. Purif. 90, 104–16. doi:10.1016/j.pep.2013.05.009

Liu, Y.K., Yang, C.J., Liu, C.L., Shen, C.R., Shiau, L.D., 2010. Using a fed-batch culture strategy to enhance rAAV production in the baculovirus/insect cell system. J. Biosci. Bioeng. 110, 187–193.

Long, G., Pan, X., Kormelink, R., Vlak, J.M., 2006. Functional entry of baculovirus into insect and mammalian cells is dependent on clathrin-mediated endocytosis. J. Virol. 80, 8830–3.

Lowy, D.R., Schiller, J.T., 2006. Prophylactic human papillomavirus vaccines. J. Clin. Invest.

116, 1167–1173.
Lu, H., Chen, Y., Liu, H., 2012. Baculovirus as a vaccine vector. Bioengineered 271–274.
Maranga, L., Cruz, P.E., Aunins, J.G., Carrondo, M.J.T., 2002. Production of core and virus-like particles with baculovirus infected insect cells. Adv. Biochem. Eng. Biotechnol. 74, 183–206.
Mena, J.A., Aucoin, M.G., Montes, J., Chahal, P.S., Kamen, A.A., 2010. Improving adeno-associated vector yield in high density insect cell cultures. J. Gene Med. 157–167.
Mitta, B., Rimann, M., Fussenegger, M., 2005. Detailed design and comparative analysis of protocols for optimized production of high-performance HIV-1-derived lentiviral particles. Metab. Eng. 7, 426–36.
Monteiro, F., Bernal, V., Saelens, X., Lozano, A.B., Bernal, C., Sevilla, A., Carrondo, M.J.T., Alves, P.M., 2014. Metabolic profiling of insect cell lines: Unveiling cell line determinants behind system's productivity. Biotechnol. Bioeng. 111, 816–28.
Monteiro, F., Carinhas, N., Carrondo, M.J.T., Bernal, V., Alves, P.M., 2012. Toward system-level understanding of baculovirus-host cell interactions: from molecular fundamental studies to large-scale proteomics approaches. Front. Microbiol. 3, 391.
Moran, N., 2012. First gene therapy nears landmark European market authorization. Nat. Biotechnol. 30, 807–9. doi:10.1038/nbt0912-807
Nguyen, B., Jarnagin, K., Williams, S., Chan, H., Barnett, J., 1993. Fed-batch culture of insect cells: a method to increase the yield of recombinant human nerve growth factor (rhNGF) in the baculovirus expression system. J. Biotechnol. 31, 205–217.
Palmberger, D., Klausberger, M., Grabherr, R., 2013. MultiBac turns sweet. Bioengineered 4, 78–83.
Palomares, L.A., López, S., Ramírez, O.T., 2004. Utilization of oxygen uptake rate to assess the role of glucose and glutamine in the metabolism of infected insect cell cultures. Biochem. Eng. J. 19, 87–93.
Palomares, L.A., Mena, J.A., Ramirez, O.T., 2012. Simultaneous expression of recombinant proteins in the insect cell-baculovirus system: Production of virus-like particles. Methods 56, 389–395.
Pijlman, G.P., van den Born, E., Martens, D.E., Vlak, J.M., 2001. Autographa californica baculoviruses with large genomic deletions are rapidly generated in infected insect cells. Virology 283, 132–138. doi:10.1006/viro.2001.0854
Pijlman, G.P., van Schinjndel, J.E., Vlak, J.M., 2003. Spontaneous excision of BAC vector sequences from bacmid-derived baculovirus expression vectors upon passage in insect cells. J. Gen. Virol. 84, 2669–2678. doi:10.1099/vir.0.19438-0
Pillay, S., Meyers, A., Williamson, A.L., Rybicki, E.P., 2009. Optimization of chimeric HIV-1 virus-like particle production in a baculovirus-insect cell expression system, in:

443 Biotechnology Progress. pp. 1153–1160.
444 Raina, A., Tuomi, K., Mäntyjärvi, R., 1981. Roles of polyamines in the replication of animal
445 viruses. Med. Biol. 59, 428–432.
446 Rodrigues, A.F., Carmo, M., Alves, P.M., Coroadinha, A.S., 2009. Retroviral vector production
447 under serum deprivation: The role of lipids. Biotechnol. Bioeng. 104, 1171–81.
448 Rodrigues, A.F., Formas-Oliveira, A.S., Bandeira, V.S., Alves, P.M., Hu, W.S., Coroadinha,
449 A.S., 2013. Metabolic pathways recruited in the production of a recombinant enveloped
450 virus: mining targets for process and cell engineering. Metab. Eng. 20, 131–45.
451 Roldão, A., Oliveira, R., Carrondo, M.J.T., Alves, P.M., 2009. Error assessment in recombinant
452 baculovirus titration: evaluation of different methods. J. Virol. Methods 159, 69–80.
453 Simons, K., Ikonen, E., 1997. Functional rafts in cell membranes. Nature 387, 569–572.
454 Taticek, R.A., Shuler, M.L., 1997. Effect of elevated oxygen and glutamine levels on foreign
455 protein production at high cell densities using the insect cell-baculovirus expression
456 system. Biotechnol. Bioeng. 54, 142–52.
457 Vieira, H.L.A., Estevao, C., Roldao, A., Peixoto, C.C., Sousa, M.F.Q., Cruz, P.E., Carrondo,
458 M.J.T., Alves, P.M., 2005. Triple layered rotavirus VLP production: Kinetics of vector
459 replication, mRNA stability and recombinant protein production. J. Biotechnol. 120, 72–
460 82.
461 Wallace, H.M., Fraser, A. V, Hughes, A., 2003. A perspective of polyamine metabolism.
462 Biochem. j. 14, 1–14.
463 Wang, Y., Oberley, L.W., Howe, D., Jarvis, D.L., Chauhan, G., Murhammer, D.W., 2004.
464 Effect of expression of manganese superoxide dismutase in baculovirus-infected insect
465 cells. Appl. Biochem. Biotechnol. 119, 181–93.
466 Wang, Y., Oberley, L.W., Murhammer, D.W., 2001. Evidence of oxidative stress following the
467 viral infection of two lepidopteran insect cell lines. Free Radic. Biol. Med. 31, 1448–1455.
468 Yang, J., Gecik, P., Collins, A., Czarnecki, S., Hsu, H., Lasdun, A., Sundaram, R.,
469 Muthukumar, G., Silberklangs, M., 1996. Rational Scale-Up of a Baculovirus-Insect Cell
470 Batch Process Based on Medium Nutritional Depth. Biotechnol. Bioeng. 52, 696–706.
471 Ylä-Herttuala, S., 2012. Endgame: Glybera Finally Recommended for Approval as the First
472 Gene Therapy Drug in the European Union. Mol. Ther.
473 Yuan, M., Huang, Z., Wei, D., Hu, Z., Yang, K., Pang, Y., 2011. Identification of Autographa
474 californica nucleopolyhedrovirus ac93 as a core gene and its requirement for intranuclear
475 microvesicle formation and nuclear egress of nucleocapsids. J. Virol. 85, 11664–74.
476 Zhang, S.X., Han, Y., Blissard, G.W., 2003. Palmitoylation of the Autographa californica
477 Multicapsid Nucleopolyhedrovirus Envelope Glycoprotein GP64 : Mapping , Functional
478 Studies , and Lipid Rafts. J. Virol. 77, 6265–6273. doi:10.1128/JVI.77.11.6265
479

## List of Tables

**Table 1.** List of culture medium supplements

| Supplement | Abbreviation | Stock concentration | Concentration added[a] | Supplier | Reference |
|---|---|---|---|---|---|
| Antioxidants | AOx | 1000 x | 1 x | Sigma (Steinheim, Germany) | A1345 |
| Biotin | Bio | 1 mM | 10 µM | Sigma | B4639 |
| Cholesterol | Chol | 250 x | 1 x | Gibco (Glasgow, UK) | 12531-018 |
| Lipids | Lip | 100 x | 1 x | Gibco | 11905-031 |
| Nucleosides | Nucl | 100 x | 1 x | Merck Millipore | ES-008-D |
| Polyamines | Poly | 1000 x | 1 x | Sigma | P8483 |
| Reduced glutathione | GSH | 100 mM | 1 mM | Sigma | G1404 |
| Taurine | Tau | 100 mM | 10 mM | Sigma | T8691 |

The supplements were added in a two times addition mode (at inoculation and upon infection). [a]Concentration of supplements added in each addition timing (inoculation and infection).

**Table 2.** Effect of media supplements on Sf9 cell growth

| Supplement | µ[a] | PDT[b] |
|---|---|---|
| Control | 0.030 ± 0.001 | 23 ± 1 |
| GSH | 0.016** ± 0.001 | 43** ± 2 |
| Antioxidants | 0.010** ± 0.003 | 69** ± 35 |
| Polyamines | 0.008** ± 0.002 | 87 ± 20 |
| Antioxidants + Polyamines | 0.014** ± 0.004 | 50 ± 22 |
| Taurine | 0.020 ± 0.003 | 35 ± 6 |
| Nucleosides | 0.029 ± 0.004 | 24 ± 3 |
| Biotin | 0.016* ± 0.003 | 43 ± 7 |
| Lipids | 0.027 ± 0.002 | 26 ± 2 |
| Cholesterol | 0.000* ± 0.003 | N.A. |

Units - [a]µ - Specific growth rate ($h^{-1}$); [b]PDT - Population doubling time (h).
The growth rate and PDT were calculated in the exponential phase of growth of non-infected cells after 24 hours incubation with the indicated supplements. Values are shown as average ± SD (n=3). N.A.- Not applicable.
Statistical significance: *p value<0.05; **p value<0.01.

**Table 3.** Summary of the effect of the supplementation strategy in bioreactor cultures on the final quality of the baculovirus stock produced.

| | Supplemented culture (Cholesterol+Polyamines) | | | | | Control culture (non-supplemented) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Time (hpi) | 3 | 24 | 48 | 72 | 96 | 3 | 24 | 48 | 72 | 96 |
| TP($10^2$.cell$^{-1}$) | 0.8 ± 0.08 | 1.6 ± 0.2 | 3 ± 0.3 | 2.8 ± 0.28 | 4 ± 0.4 | 1.9 ± 0.2 | 2.6 ± 0.3 | 4.4 ± 0.4 | 5.2 ± 0.5 | 8.6 ± 0.7 |
| IP($10^{-1}$.cell$^{-1}$) | 0.8 ± 0.2 | 1.3 ± 0.4 | 3.2 ± 1 | 5.5 ± 2 | 6.9 ± 2 | 1.0 ± 0.3 | 0.8 ± 0.2 | 1.0 ± 0.3 | 1.6 ± 0.5 | 1.6 ± 0.5 |
| Ratio TP/IP | $1.1x10^3$ | $1.3x10^3$ | $9.1x10^2$ | $5.1x10^2$ | $5.7x10^2$ | $1.9x10^3$ | $3.2x10^3$ | $4.4x10^3$ | $3.2x10^3$ | $5.3x10^3$ |
| AF[a] | 7 | 18 | 69 | 128 | 124 | 10 | 7 | 8 | 21 | 26 |

[a]The amplification factor (AF) was calculated as the ratio between the volumetric productivity and the number of infectious baculovirus per mL at the time of infection (volumetric productivity/(CCI×MOI)).

**List of Figure Captions:**

**Figure 1. Schematic representation of the experimental workflow.** The exploratory screening of culture supplements **(A)** was performed in the TAP Ambr system, the extensive screening **(B)** and the analysis of the synergistic effects **(C)** were performed in Erlenmeyers and the scale-up **(D)** in 0.5 L stirred tank bioreactors. The culture conditions are shown, including the MOIs used and the supplements added to cultures. Abbreviations: GSH, reduced glutathione; AOx, antioxidants; Poly, polyamines; Tau, taurine; Nucl, nucleosides; Bio, biotin; Lip, lipids; Chol, cholesterol.

**Figure 2. Extensive screening of cell culture supplements for target-oriented bioprocess optimization in Sf9 cells. (A)** Infectious baculovirus particles (IPs), and **(B)** Influenza VLPs (Inf-VLPs). The results represent the specific yield of IPs and Inf-VLPs, on a *per* cell basis. Infections were performed at CCI of $1x10^6$ cells.mL$^{-1}$ varying the MOI as indicated in the bottom axes. The data shown correspond to 48 hpi; error bars indicate variability between two independent supplemented cultures and three independent control cultures.

**Figure 3. Effect of cell culture supplements on viral stock quality.** Upper panel: Total to infectious particles content ratio (TP/IP). Bottom panel: Specific yields of total (black bars) and infectious (light grey bars) viral particles produced in Sf9 cells. The left axis represents the quantification of baculovirus TPs, and the right axis the quantification of baculovirus IPs. Infections were performed at a CCI of $1x10^6$ cells.mL$^{-1}$ with an MOI of 0.2 IP·cell$^{-1}$. The data shown correspond to 48 hpi; error bars indicate variability between two independent supplemented cultures or three independent control cultures.

**Figure 4. Synergistic effect of cell culture supplements on the specific yields of baculovirus IPs and Inf-VLPs produced in Sf9 cells.** Dark grey bars correspond to infectious baculovirus titers and light grey bars to Influenza VLPs specific productivities. The results are shown as fold-change on the specific yields of supplemented *versus* control (non-supplemented) cultures. Infections were performed at a CCI of $1x10^6$ cells.mL$^{-1}$ with an MOI of 0.2 IP·cell$^{-1}$. The data shown correspond to 48 hpi; error bars assume 30% of inter-assay variability for infectious baculovirus and 10% for influenza VLPs quantification. Control cultures were performed in triplicates. N.C.- No change.

**Figure 5. Improved production and quality of enveloped viral particles in Sf9 cells: Validation of the best supplementation scheme in stirred-tank bioreactors. (A)** Cell specific yields of infectious baculovirus in control (◇) and supplemented cultures (◆); **(B)** Cell Specific yields of Influenza VLPs in control (○) and supplemented (●) cultures**.** The results show the bioprocess timeline after infection. Cultures were performed in a Biostat bioreactor, as

described in the Materials and Methods section. Infections were performed at a CCI of $1x10^6$ cells.mL$^{-1}$ with an MOI of 0.2 IP·cell$^{-1}$. The supplementation scheme comprised the addition of a combination of polyamines and cholesterol at the times of inoculation and infection, as described in the M&M section. Error bars correspond to the variability of technical replicates.

**List of Tables and Figures:**

## List of Tables:

| Supplement | Abbreviation | Stock concentration | Concentration added[a] | Supplier | Reference |
|---|---|---|---|---|---|
| Antioxidants | AOx | 1000 x | 1 x | Sigma (Steinheim, Germany) | A1345 |
| Biotin | Bio | 1 mM | 10 µM | Sigma | B4639 |
| Cholesterol | Chol | 250 x | 1 x | Gibco (Glasgow, UK) | 12531-018 |
| Lipids | Lip | 100 x | 1 x | Gibco | 11905-031 |
| Nucleosides | Nucl | 100 x | 1 x | Merck Millipore | ES-008-D |
| Polyamines | Poly | 1000 x | 1 x | Sigma | P8483 |
| Reduced glutathione | GSH | 100 mM | 1 mM | Sigma | G1404 |
| Taurine | Tau | 100 mM | 10 mM | Sigma | T8691 |

**Table 1.** List of culture medium supplements

The supplements were added in a two times addition mode (at inoculation and upon infection). [a]Concentration of supplements added in each addition timing (inoculation and infection).

**Table 2.** Effect of media supplements on Sf9 cell growth

| Supplement | μ[a] | PDT[b] |
|---|---|---|
| Control | 0.030 ± 0.001 | 23 ± 1 |
| GSH | 0.016** ± 0.001 | 43** ± 2 |
| Antioxidants | 0.010** ± 0.003 | 69** ± 35 |
| Polyamines | 0.008** ± 0.002 | 87 ± 20 |
| Antioxidants + Polyamines | 0.014** ± 0.004 | 50 ± 22 |
| Taurine | 0.020 ± 0.003 | 35 ± 6 |
| Nucleosides | 0.029 ± 0.004 | 24 ± 3 |
| Biotin | 0.016* ± 0.003 | 43 ± 7 |
| Lipids | 0.027 ± 0.002 | 26 ± 2 |
| Cholesterol | 0.000* ± 0.003 | N.A. |

Units - [a]μ - Specific growth rate ($h^{-1}$); [b]PDT - Population doubling time (h).
The growth rate and PDT were calculated in the exponential phase of growth of non-infected cells after 24 hours incubation with the indicated supplements. Values are shown as average ± SD (n=3). N.A.- Not applicable.
Statistical significance: *p value<0.05; **p value<0.01.

**Table 3.** Summary of the effect of the supplementation strategy in bioreactor cultures on the final quality of the baculovirus stock produced.

| | Supplemented culture (Cholesterol+Polyamines) | | | | | Control culture (non-supplemented) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Time (hpi) | 3 | 24 | 48 | 72 | 96 | 3 | 24 | 48 | 72 | 96 |
| TP($10^2$.cell$^{-1}$) | 0.8 ± 0.08 | 1.6 ± 0.2 | 3 ± 0.3 | 2.8 ± 0.28 | 4 ± 0.4 | 1.9 ± 0.2 | 2.6 ± 0.3 | 4.4 ± 0.4 | 5.2 ± 0.5 | 8.6 ± 0.7 |
| IP($10^{-1}$.cell$^{-1}$) | 0.8 ± 0.2 | 1.3 ± 0.4 | 3.2 ± 1 | 5.5 ± 2 | 6.9 ± 2 | 1.0 ± 0.3 | 0.8 ± 0.2 | 1.0 ± 0.3 | 1.6 ± 0.5 | 1.6 ± 0.5 |
| Ratio TP/IP | $1.1x10^3$ | $1.3x10^3$ | $9.1x10^2$ | $5.1x10^2$ | $5.7x10^2$ | $1.9x10^3$ | $3.2x10^3$ | $4.4x10^3$ | $3.2x10^3$ | $5.3x10^3$ |
| AF[a] | 7 | 18 | 69 | 128 | 124 | 10 | 7 | 8 | 21 | 26 |

[a]The amplification factor (AF) was calculated as the ratio between the volumetric productivity and the number of infectious baculovirus per mL at the time of infection (volumetric productivity/(CCI×MOI)).

**Figure 1.**

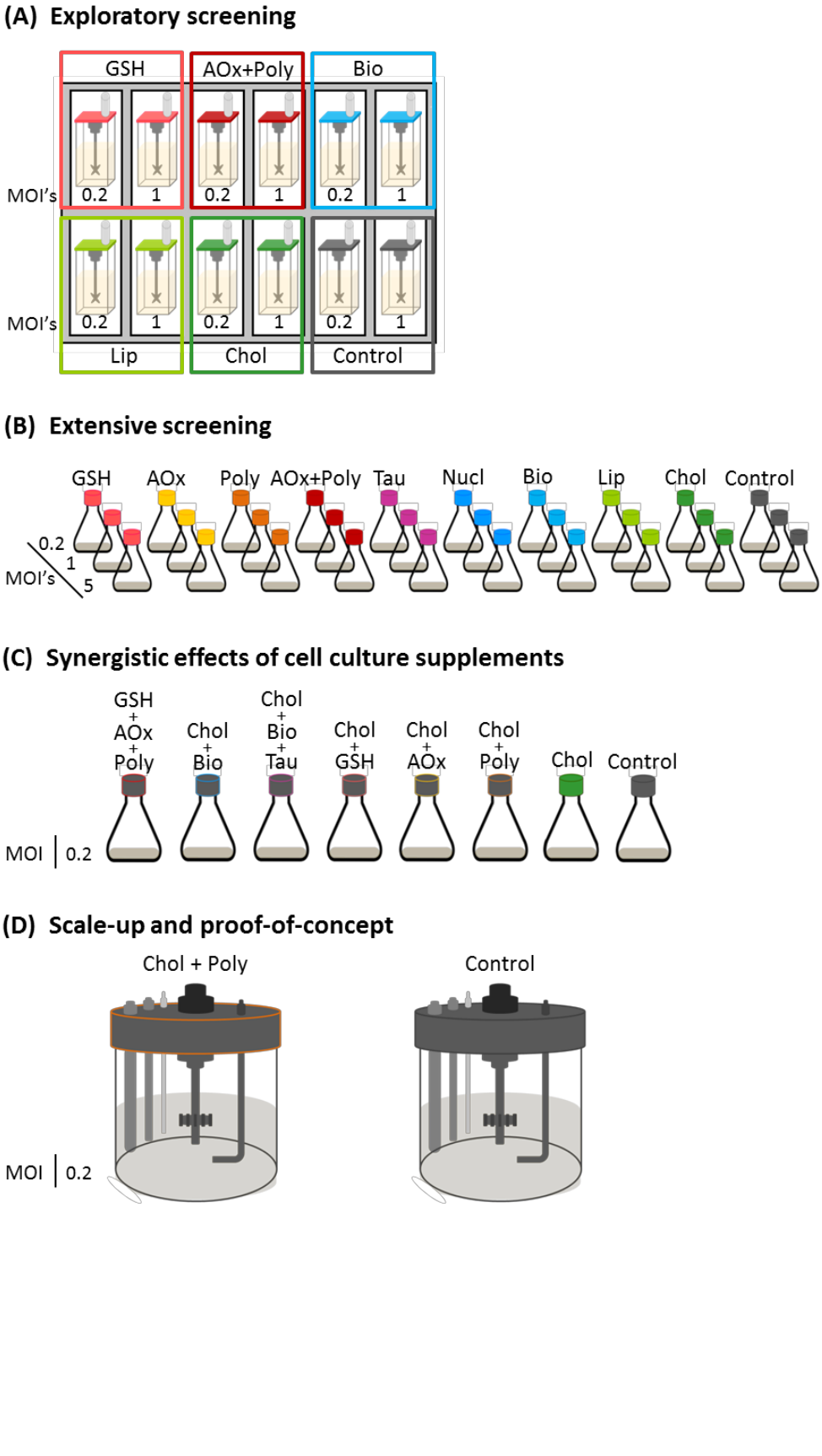

(A) Exploratory screening
GSH
AOx+Poly
Bio
MOI's
0.2
1
Lip
Chol
Control
(B) Extensive screening
GSH
AOx
Poly
AOx+Poly
Tau
Nucl
Bio
Lip
Chol
Control
MOI's
0.2
1
5
(C) Synergistic effects of cell culture supplements
GSH + AOx + Poly
Chol + Bio
Chol + Bio + Tau
Chol + GSH
Chol + AOx
Chol + Poly
Chol
Control
MOI
0.2
(D) Scale-up and proof-of-concept
Chol + Poly
Control
MOI
0.2

**Figure 2.**

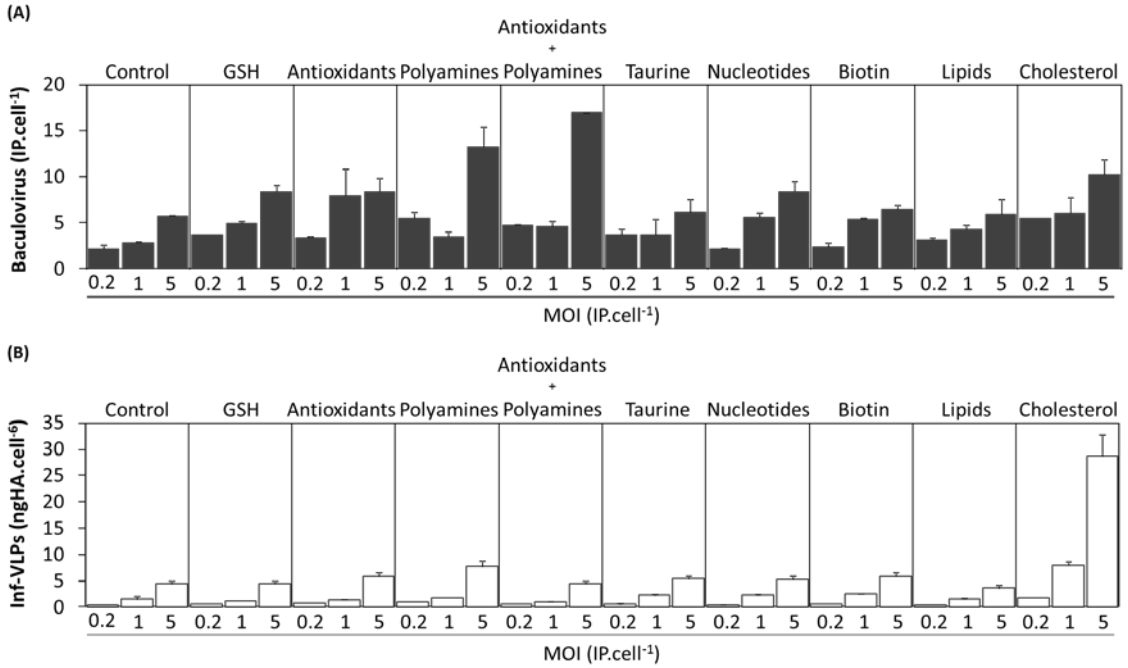

(A)
Antioxidants
+
Control
GSH
Antioxidants
Polyamines
Polyamines
Taurine
Nucleotides
Biotin
Lipids
Cholesterol
Baculovirus (IP.cell-1)
0
5
10
15
20
0.2 1 5 0.2 1 5 0.2 1 5 0.2 1 5 0.2 1 5 0.2 1 5 0.2 1 5 0.2 1 5 0.2 1 5 0.2 1 5
MOI (IP.cell-1)
(B)
Antioxidants
+
Control
GSH
Antioxidants
Polyamines
Polyamines
Taurine
Nucleotides
Biotin
Lipids
Cholesterol
Inf-VLPs (ngHA.cell-6)
0
5
10
15
20
25
30
35
0.2 1 5 0.2 1 5 0.2 1 5 0.2 1 5 0.2 1 5 0.2 1 5 0.2 1 5 0.2 1 5 0.2 1 5 0.2 1 5
MOI (IP.cell-1)

**Figure 3.**

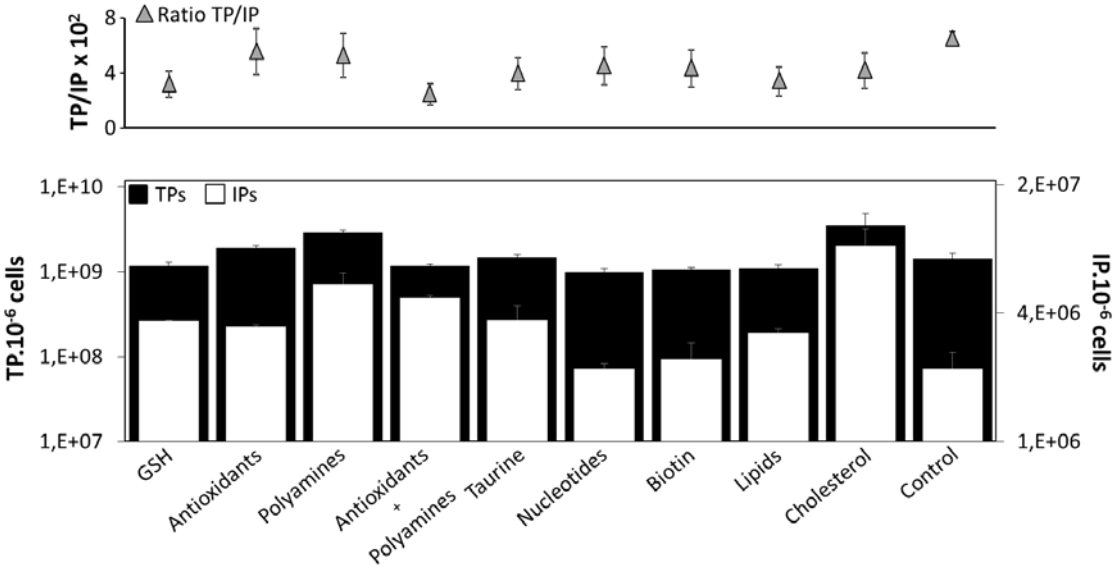

Ratio TP/IP
TP/IP x 10²
8
4
0
TPs
IPs
1,E+10
1,E+09
1,E+08
1,E+07
TP.10⁻⁶ cells
2,E+07
4,E+06
1,E+06
IP.10⁻⁶ cells
GSH
Antioxidants
Polyamines
Antioxidants + Polyamines
Taurine
Nucleotides
Biotin
Lipids
Cholesterol
Control

**Figure 4.**

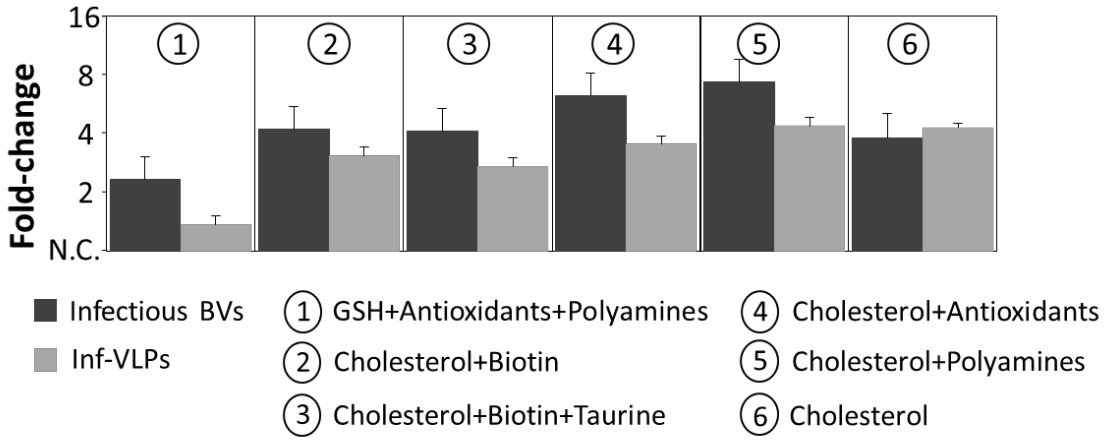
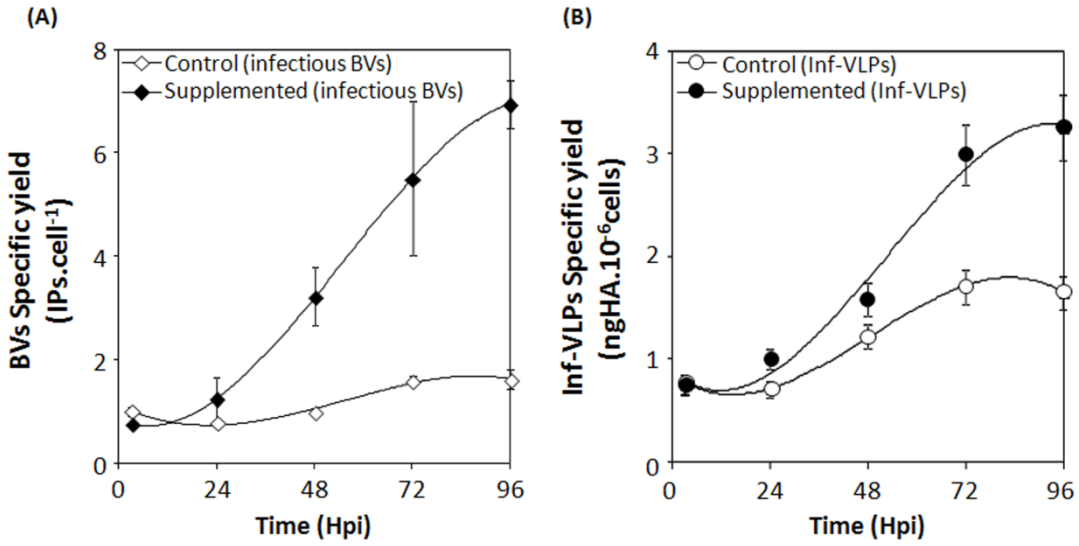

16
8
4
2
N.C.
Fold-change
1
2
3
4
5
6
Infectious BVs
Inf-VLPs
1 GSH+Antioxidants+Polyamines
2 Cholesterol+Biotin
3 Cholesterol+Biotin+Taurine
4 Cholesterol+Antioxidants
5 Cholesterol+Polyamines
6 Cholesterol

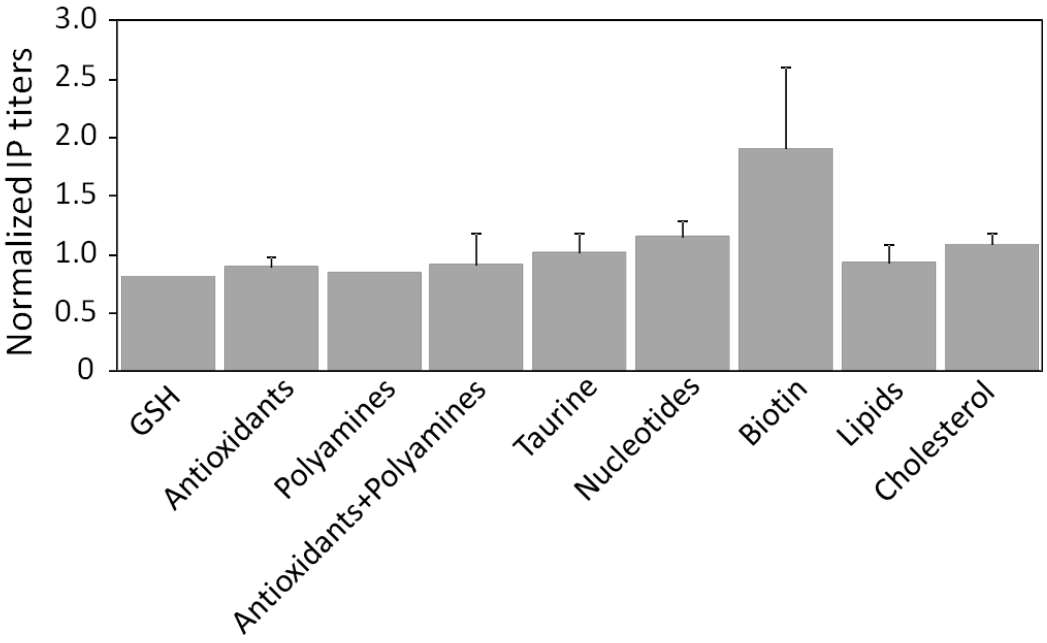
**Figure 5.**


(A)
Control (infectious BVs)
Supplemented (infectious BVs)
BVs Specific yield (IPs.cell-1)
Time (Hpi)
(B)
Control (Inf-VLPs)
Supplemented (Inf-VLPs)
Inf-VLPs Specific yield (ngHA.10-6cells)
Time (Hpi)

**Supplementary Table 1.** Impact of media supplements on the productivity of enveloped viral particles in Sf9 cells

| Supplement | MOI | Inf-VLP Volumetric titer[a] | Inf-VLP Yield[b] | BVs Volumetric titer[c] | BVs Yield[d] |
|---|---|---|---|---|---|
| Control | 0.2 | 0.45 ± 0.06 | 0.40 ± 0.1 | 1.98 ± 0.75 | 1.78 ± 0.09 |
| | 1 | 0.99 ± 0.53 | 1.52 ± 0.6 | 2.90 ± 0.49 | 2.89 ± 0.14 |
| | 5 | 4.38 ± 0.59 | 4.52 ± 0.4 | 6.12 ± 0.62 | 5.69 ± 0.19 |
| GSH | 0.2 | 0.56 ± 0.02 | 0.63 ± 0.03 | 3.32 ± 0.00 | 3.70 ± 0.00 |
| | 1 | 1.01 ± 0.10 | 1.13 ± 0.11 | 4.44 ± 0.21 | 4.97 ± 0.24 |
| | 5 | 3.85 ± 0.53 | 4.39 ± 0.60 | 7.39 ± 0.54 | 8.43 ± 0.62 |
| Antioxidants | 0.2 | 0.59 ± 0.02 | 0.76 ± 0.03 | 2.67 ± 0.05 | 3.47 ± 0.07 |
| | 1 | 1.13 ± 0.11 | 1.42 ± 0.14 | 6.35 ± 2.31 | 7.97 ± 2.90 |
| | 5 | 5.11 ± 0.70 | 5.72 ± 0.78 | 7.52 ± 1.28 | 8.42 ± 1.43 |
| Polyamines | 0.2 | 0.57 ± 0.02 | 0.86 ± 0.04 | 3.65 ± 0.44 | 5.50 ± 0.66 |
| | 1 | 1.37 ± 0.14 | 1.64 ± 0.16 | 2.90 ± 0.47 | 3.48 ± 0.56 |
| | 5 | 4.48 ± 0.61 | 7.77 ± 1.06 | 7.67 ± 1.19 | 13.29 ± 2.06 |
| Antioxidants + Polyamines | 0.2 | 0.52 ± 0.02 | 0.51 ± 0.02 | 4.80 ± 0.13 | 4.71 ± 0.13 |
| | 1 | 1.01 ± 0.10 | 1.04 ± 0.10 | 4.48 ± 0.50 | 4.65 ± 0.52 |
| | 5 | 4.47 ± 0.61 | 4.49 ± 0.61 | 16.99 ± 0.15 | 17.05 ± 0.15 |
| Taurine | 0.2 | 0.66 ± 0.03 | 0.65 ± 0.03 | 3.78 ± 0.62 | 3.72 ± 0.61 |
| | 1 | 2.89 ± 0.29 | 2.34 ± 0.23 | 4.68 ± 1.98 | 3.80 ± 1.60 |
| | 5 | 5.39 ± 0.74 | 5.33 ± 0.73 | 6.28 ± 1.34 | 6.21 ± 1.32 |
| Nucleosides | 0.2 | 0.50 ± 0.02 | 0.46 ± 0.02 | 2.38 ± 0.12 | 2.20 ± 0.11 |
| | 1 | 2.34 ± 0.23 | 2.27 ± 0.23 | 5.82 ± 0.61 | 5.63 ± 0.42 |
| | 5 | 5.28 ± 0.72 | 5.27 ± 0.72 | 8.42 ± 1.08 | 8.39 ± 1.07 |
| Biotin | 0.2 | 0.60 ± 0.03 | 0.59 ± 0.02 | 2.50 ± 0.47 | 2.44 ± 0.46 |
| | 1 | 2.67 ± 0.27 | 2.46 ± 0.25 | 5.89 ± 0.11 | 5.44 ± 0.11 |
| | 5 | 5.74 ± 0.79 | 5.76 ± 0.79 | 6.51 ± 0.46 | 6.53 ± 0.46 |
| Lipids | 0.2 | 0.45 ± 0.02 | 0.41 ± 0.02 | 3.57 ± 0.14 | 3.25 ± 0.12 |
| | 1 | 2.08 ± 0.21 | 1.62 ± 0.16 | 5.52 ± 0.50 | 4.30 ± 0.39 |
| | 5 | 4.02 ± 0.55 | 3.73 ± 0.51 | 6.36 ± 1.73 | 5.91 ± 1.61 |
| Cholesterol | 0.2 | 0.53 ± 0.02 | 1.67 ± 0.07 | 4.35 ± 2.60 | 8.29 ± 1.64 |
| | 1 | 2.86 ± 0.29 | 7.95 ± 0.79 | 2.18 ± 0.61 | 6.06 ± 1.69 |
| | 5 | 12.16 ± 1.67 | 28.73 ± 3.94 | 4.37 ± 0.64 | 10.33 ± 1.52 |

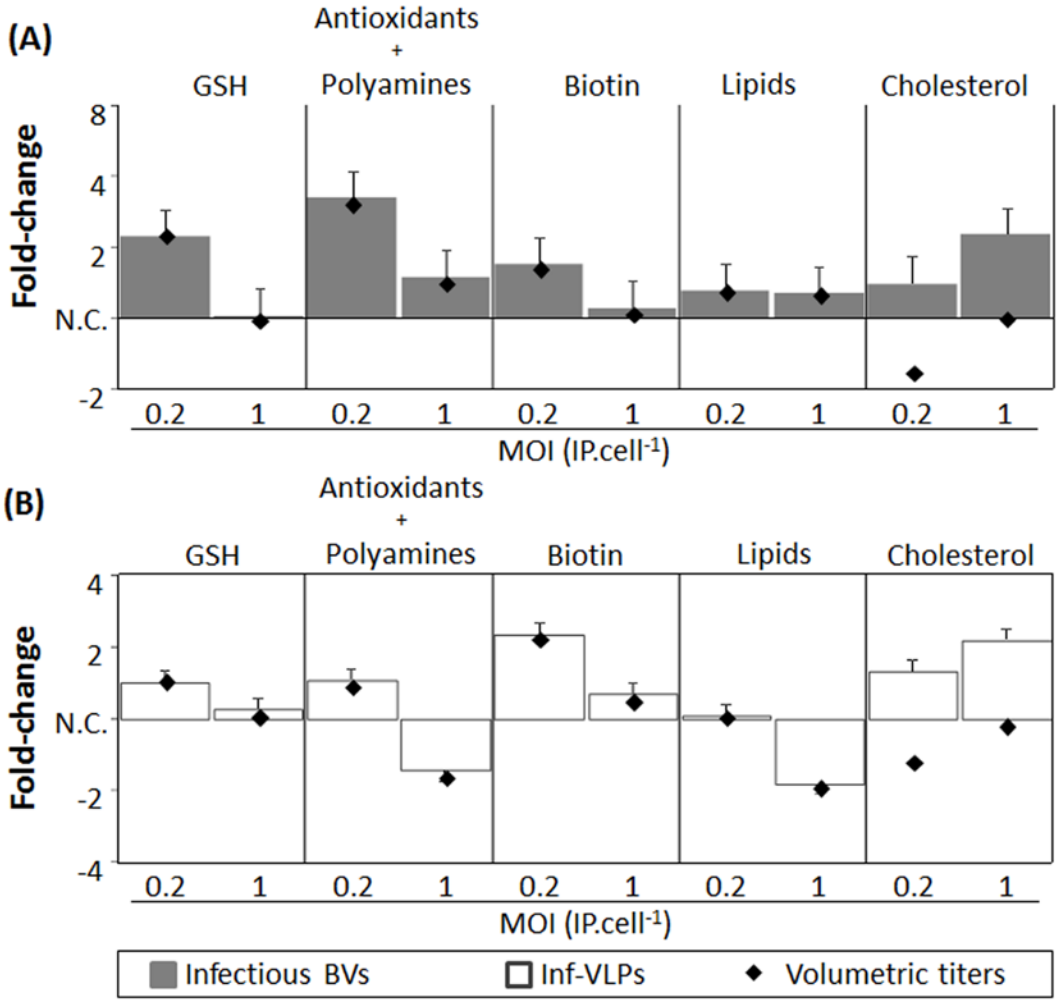
Units - [a]ngHA.mL$^{-1}$; [b]ngHA.10$^{-6}$cells; [c]10$^{6}$IPs.mL$^{-1}$; [d]IPs.cell$^{-1}$.
Values represent the mean average and variability of two independent replicates; samples were collected at 48 hpi and analyzed for the production of Inf-VLPs and infectious BVs (IP).




782

783 **Supplementary Figure 1.** Stability of infectious baculovirus in the presence of media
784 supplements. Titers were normalized by the control (non-supplemented baculovirus sample)
785 titer.

786




787

788 **Supplementary Figure 2.** Preliminary screening of the impact of culture supplements on the
789 specific yields of Sf9 cells. **(A)** Infectious baculovirus, BVs (grey bars); **(B)** Influenza VLPs,
790 Inf-VLPs (white bars). The results are presented as fold-change in the specific yields of
791 supplemented *versus* control (non-supplemented) cultures. Dots indicate the fold-change in the
792 volumetric titers of the target products analyzed. Cultures were performed in the Ambr system,
793 as described in the M&M section. Infections were performed at a CCI of $1x10^6$ cells.$mL^{-1}$
794 varying the MOI as indicated. The data corresponds to 48 hpi; error bars correspond to 30% of
795 inter-assay variability for infectious BVs and 10% for Inf-VLPs quantification. Error bars for
796 volumetric titers were ommitted for simplicity reasons. N.C. - No change.

797